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PCR-RFLP identification of trichodorid nematodes

Molecular diagnosis of trichodorid species from Portugal

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Abstract

A PCR-RFLP assay was developed for the identification of trichodorids belonging to *Nanidorus*, *Paratrichodorus* and *Trichodorus* genera. Exploring the variability of the 18S SSU rDNA gene this method provides for the first time a molecular diagnosis tool alternative to the difficult and time consuming phenotyping especially of quite similar species, enabling selectivity in mixed samples with non-trichodorid species, and also the differentiation of juveniles. Based on the alignment of previously obtained 18S rDNA nucleotide sequences of trichodorids from Portugal, a pair of selective primers was designed in conserved regions to allow the amplification in all known Portuguese species of a variable region located at the 3' end of the gene. The PCR product, 615bp in length, exhibits nucleotide variability to generate restriction fragment patterns which were consistent among populations of the same species and allowed to discriminate trichodorids at the species level. The proposed protocol was tested and proved effective with twelve trichodorid species from Portugal (*N. minor*, *P. allius*, *P. anemones*, *P. divergens*, *P. hispanus*, *P. pachydermus*, *P. porosus*, *T. beirensis*, *T. lusitanicus*, *T. primitivus* and two other *Trichodorus* species, A and B) and six non-indigenous trichodorid populations (*N. minor*, *P. allius*, *P. anemones*, *P. pachydermus*, *P. porosus*, *T. primitivus*).

Keywords – identification, intraspecific variation, PCR-RFLP, ribosomal DNA, trichodorid, 18S sequence.

Introduction

Didelphic *Nanidorus*, *Paratrichodorus* and *Trichodorus* nematodes of Trichodoridae family are economically important polyphagous ectomigratory plant root ectoparasites. More than 90 species are described (Almeida & Decraemer, 2005), some being known to cause direct damage by feeding, resulting in a reduction of root development, and a few (13) are vector specific strains of TRV, *Pea early-browning virus* and *Pepper ringspot virus*, that have a deleterious effect upon several economically important crops, such as ornamental bulbs, potatoes and tobacco (Harrison & Robinson, 1978, 1986; Taylor & Brown, 1997). Their distribution is wide in North America and Europe, but they have been reported from different parts of the world (Taylor & Brown, 1997). Up to now, sixteen trichodorid species have been reported in Portugal (Almeida *et al.*, 1989; Almeida & Santos, 1997), some known as TRV vectors (Brown & Weischer, 1998; Duarte *et al*., 2001, 2002, 2010).

Tobraviruses have a wide host range, basically annual plants that can perennate the virus (Cooper & Harrison, 1973) and vector nematodes, in which can be retained for months or years (van Hoof, 1970). Infected roots and vectors act as virons reservoirs providing inocula to be gradually transmitted, by a compatible vector, to the roots of healthy susceptible adjacent plants. These features play an effective role in the tobravirus dissemination, bridging the infection between successive susceptible crops and enable the virus to be exposed to their host range, finding potential hosts in different plants (Harrison, 1977; Harrison & Robinson, 1978, 1986).

The commercial activities of plant material have favoured the dispersal of both agents, conducting to more diverse encounters between trichodorid species and virus isolates. When field populations of trichodorids become viruliferous, they can persist infected for many years (Harrison, 1977, Weingartner, 2001), being known to cause high economical losses and in growing areas of susceptible crops (Mojathedi *et al.,* 2000, 2001; Weingartner, 2001), mainly due to its difficult control (Ploeg & Decraemer, 1997). Presently, the difficulties of disease management in fields already contaminated are related with the efficient virus survival strategy,

the inexistence of diverse crop cultivars TRV resistant, and the lack of efficient chemical treatments, as nematicides do not eradicate the problem and have high environmental containments upon their use.

Given the complex relationship that exists between trichodorids and the various TRV strains (Brown & Weischer, 1998) and the limitations of the currently available control tools, it is essential to prevent the introduction of TRV to virus-free areas and to minimise the problem in infected regions, for which it is crucial a rapid and accurate identification of trichodorids to the species level. Currently, this identification is based on morphobiometrical characterization, which requires the drawing and measurement of body structures under x1000 amplification of several adult specimens of each population, which is a laborious and time-consuming task that requires expertise services. The differentiation of some species, mainly in *Paratrichodorus*, is difficult due to high intraspecific variability. Very frequently in the growing season, *e.g*. at sampling time, small numbers of specimens, or juveniles are the only stage present, commonly in a complex mixture of vector and non-vector species. Without the reproductive structures, which are main diagnostic features for morphometrical identification, immature specimens are difficult or impossible to identify. For crop protection management, it is however, important to identify those immature stages at species level. Furthermore, with the decline of skill-base nematode taxonomists within Europe, there is an urgent demand for the development of an alternative tool for a routine detection and clear identification of trichodorids.

Direct-analysis of the genetic material is potentially a powerful method to identify nematodes. Classification of plant parasite nematodes based on the PCR amplification (Saiki *et al*., 1988) of specific DNA regions is nowadays a routine. For these purposes, mitochondrial DNA (Powers & Harris, 1993; Adam *et al*., 2007; Kumari & Lišková, 2009), satellite DNA (Castagnone-Sereno *et al*., 1998) and the ITS region of ribosomal DNA (Zijlstra *et al*., 1995, 1997; Iwahori *et al*., 1998; Wang *et al*., 2003; Hübschen *et al*., 2004a, b; Berry *et al*., 2007, 2008) are widely used. The ITS and 18S regions have been also exploit to resolve nematodes phylogenetic relationships (Blaxter *et al*., 1998; Megen *et al*., 2009; Duarte *et al*., 2010), for DNA barcode-based nematode

detection and biodiversity analysis (Floyd *et al*., 2005) and successfully applied to identification of *Xiphidorus* spp. (Oliveira *et al*., 2004), virus vector nematodes, *Longidorus* spp., *Paratrichodorus* spp., *Trichodorus* spp. and *Xiphinema* spp. (Vrain *et al*., 1992; Molinari *et al*., 1997; Boutsika *et al*., 2004; Holeva *et al*., 2006; Riga *et al*., 2007).

The aim of this work was to develop a simple and inexpensive molecular method in order to allow the identification of trichodorid species present in Portugal. An rDNA-PCR-RFLP (restriction fragment length polymorphism) was designed as a diagnostic technique that explores the variability of the 18S rDNA region to discriminate trichodorid species belonging to *Nanidorus, Paratrichodorus and Trichodorus* producing clear species-specific diagnostic patterns. The method was tested with trichodorid populations of different geographic origins.

Materials and methods

PCR-RFLP design

The selection of a genomic region within the SSU rDNA sequences, to allow a clear species differentiation, was based on the analysis of a set of nucleotide sequences previously obtained in 2010 by Duarte *et al*. (Table 1), and their deduced restriction enzymes (RE) maps, resorting to the Package programmes MAP, MAPPLOT and MAPSORT, GCG version 10·0, Wisconsin Package programmes. A computer programme was build to assist the *in vitro* design of the PCR-RFLP for this conserved region. Aiming the design a simple technique, the selection criteria obliged the use of a unique pair of trichodorid-specific primers to amplify a small region, containing nucleotide variability detectable by restriction endonuclease cleavage. The programme was run either to the entire 18S sequence or to variable sub-regions, corresponding to viable/pragmatic PCR products to select RE cleavage sites able to differentiate the set of trichodorid species under study. The REs were selected aiming the production of species-specific

digested products, differentiable in size from each other by over 80-100bp to allow their easier visualisation by electrophoresis, through agarose gels.

Nematode species

Twenty-one populations comprising 12 *Trichodorus, Paratrichodorus* and *Nanidorus* species were collected from different geographical regions in Portugal and abroad. *Trichodorus* sp. A and *Trichodorus* sp. B are designated following Almeida (1993). *Paratrichodorus* sp. C and sp. D refer to two different unidentified trichodorid species (Table 1). All nematodes were extracted directly from infested field soil (Brown & Boag, 1988) and used without subsequent cultivation. Representative adult specimens from each species were collected, morphological and morphometric identified and kept in 1M NaCl at -20ºC. Five species (*N. minor*, *P. divergens, P. pachydermus, P. porosus* and *Trichodorus* sp. A) are represented by two populations from different geographic origins while *T. primitivus* is represented with three populations.

DNA extraction and PCR amplification

Total DNA was extracted from a single adult trichodorid (Stanton *et al*., 1998) after morphological and morphometric confirmation and photomicrograph recording.

PCR reactions were performed with 10mM-Tris/HCI (pH 8·8), 50mM KCl, 1·5mM $MgCl_2$, 0·2μM of each primer, 0·2mM of d**N**TP, and 0·5-1μl of nematode total DNA (0·05 to 0·5ng DNA template) and 2·5 units of Taq DNA polymerase. The reactants for amplification were supplied by *Invitrogen, Life Technologies*. The primers used in the PCR reaction, 1091-F (5'-AGGAATTGACGGAAGGGCAC-3') and 1671-R (5'-TCCTCTAAGTAAATCCCATTGG-3') were synthesised and supplied by the same manufactor.

The thermal cycling parameters included an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 30 s, with an extension at

72°C for 2 min, and an additional final extension step at 72°C for 10 min. Negative control without DNA template and a positive control with *T. primitivus* DNA were included in all experiments. Separate specimens of each species were used to produce the PCR products. All the nucleotide sequences in this study were therefore obtained from one unique individual. All sequence analysis were done with GCG version 10·0 (Genetics Computer Group), Wisconsin Package programmes.

RFLP

Approximately 1·5-3µg of each PCR product (*ca*. 615bp of the amplicom) were single digested with 5U of *Aat*II, *Ale*I, *Ava*I, *Bsr*I, *Fnu*4HI, *Taq*I or *Tsp*RI, according to the manufacturers recommendations, in a final volume of 12µl. The digestions with *Bsr*I, *Taq*I and *Tsp*RI were conducted for 2 h at 65ºC, while the remaining four were carried out at 37ºC.

The hydrolysed PCR products were size-separated in a 2% agarose gel (Agarose I, *Stratagene*) in 1X TBE buffer (89mM Tris/HCl, 89mM boric acid, 2mM EDTA), containing 0·5µg/ml of ethidium bromide and run at *ca*. 7 V/cm at RT in 1X TBE buffer. A 100bp ladder (*Invitrogen, Life Technologies*) and a XIII (50-750bp), *Roche, Diagnostic GmbH* DNA were used as size markers. The DNA fragments were visualised by gel observation in a 300nm wavelength UV transilluminator, photographed with a Polaroid Instant Camera System and the band pattern analysed visually.

Evaluation of the molecular diagnostic method (PCR-RFLP)

Total cellular DNA was extracted from 42 separate individuals, representing 21 populations (two individuals of each population) and subjected to the proposed PCR-RFLP protocol to evaluate the technique as a species differentiator tool. Patterns obtained with DNA prepared from two individuals of each species prepared in two occasions were compared to evaluate the possible variation in PCR-RFLP patterns when obtained in different experiments.

Two parameters were used to evaluate the PCR-RFLP as a differentiation system for trichodorid species. The typability, as the percentage of organisms to which was possible to obtain a positive and non-ambiguous result with the system; and the reproducibility, as the percentage of organisms, which produce the same result, when repeatedly tested with the system.

The specificity of this technique and the putative occurrence of false positive results were tested *in silico* by resorting to several species, which sequences were published in the GenBank, at that time: sequences of *Longidorus elongates* (AF03659), *Prismatolaimus intermedius (*AF036603), *Pungentus silvestris* (AY284788), *Pungentus* sp. (AJ966501), *Thornia steatopyga* (AY284787), *Tylencholaimellus striatus* (AY284837) and *Xiphinema rivesi* (AF036610). This selection included taxonomically related genera, *Prismatolaimus*, *Longidorus* and *Xiphinema* and also those that, under the stereoscopic microscope, could be more easily confounded with trichodorids, when picking the specimens from the soil suspension for DNA extraction, namely, *Diphtherophora*, *Doryllium, Lepthonchus, Pungentus*, *Proleptonchus*, *Thornia* and *Tylencholaimellus*. The specificity of the primers, that confer the specificity of the PCR phase, was assessed against the NCBI nucleotide sequence database (05.08.2010) (ftp://ftp.ncbi.nih.gov/blast/db/FAST). Furthermore, the specificity of the restriction enzyme of the referred sequences was also investigated searching for identical restriction patterns, using MAPSORT programme.

Strictly for the purpose of this work, molecular type species were defined referring to the 18S rDNA nucleotide sequences corresponding to morphobiometrical well characterized trichodorid specimens of twelve species (Table 1). The molecular type species of *P. anemones (*AF036600) and *P. pachydermus* (AF036601) (Blaxter *et al*., 1998) were considered as reference sequences.

Results

Identification of a candidate region for PCR-RFLP design

The selected genomic region of *ca*. 615bp, located at the 3´end of the gene, fulfils the stated requirements. The BLAST search with the selected primers mach the DNA of no other organisms, being therefore, apparently trichodorid-specific, but the amplicons yielded are size undifferentiated.

Due to the low rate of variable positions in this region, the discrimination of all species was only achieved by the analysis of combined results obtained with several different simple restriction. As shown in Table 2 and Figure 1, the *Aat*II, *Ale*I, *Ava*I, *Bsr*I, *Fnu*4HI, *Taq*I and *Tsp*RI restriction enzyme maps, of the selected region within the 18S rDNA of trichodorid sequences, yield enough variability to distinguish the species under analysis.

PCR-RFLP

Amplification of the rDNA-18S region was successful to all the 42 specimens collected, generating the expected fragments with *ca*. 615bp. The detection of DNA polymorphisms by PCR-RFLP allowed the identification of all populations, confirming the predictions made from the nucleotide data analysis (Figure 1).

The technique did not discriminate populations of the same species, collected from different geographic origins, mainly from continental Portugal, which reinforces the species specificity of the method. As a matter of fact, identical patterns were observed in two different Portuguese populations of *P. divergens* and *Trichodorus* sp. A; two different populations of *N. minor* and *P. porosus* (one Portuguese and one Brazilian) and two different populations of *P. pachydermus* and *T. primitivus* (one Portuguese-continental and one Scottish). These results indicate that despite their origin, at least the 3' end of the 18S gene is totally conserved. *T. primitivus* population from Azores was included in the PCR-RFLP analysis but was not sequenced due to sample limitations. The *Fnu*4H restriction pattern of *T. primitivus* population from Azores Island

differed from both the continental and the Scottish ones by an additional band of *ca*. 270bp. As the size of the PCR fragment is conserved in this species (615bp) this difference is probably the result of partial digestion of the *Fnu*4HI site located between the contiguous fragments 192bp and 83bp.

Through *Aat*II, *Fnu*4HI and *Taq*I restriction, this technique also allows a clear distinguishing of the morphobiometrical similar *P. pachydermus* and *Paratrichodorus* sp. A populations, both from the same geographical region of Vila Real (North of Portugal) (Figure 1). The juvenile specimens of *Paratrichodorus* sp. D were collected in Chaves, where a mixed population of *P. anemones* and *P. pachydermus* had been found. Application of the PCR-RFLP technique did not generate a pattern similar to any of the patterns obtained with those other species. In fact, its RFLP pattern is identical to *P. pachydermus*, except for the *Taq*I restriction products that resemble *Paratrichodorus* sp. C (Figure 2). Further studies of that population will provide additional molecular and morphological data to clarify if these individuals are from a different molecular species, or constitute a molecular variant of *P. pachydermus*.

No single enzymatic restriction could distinguish all the species in this study, although some have much more taxonomic potential resolution than others. *Bsr*I restriction allows distinguishing *Nanidorus* and *Paratrichodorus* from *Trichodorus*, generating also a *P. porosus* specific pattern. Differentiation between *Nanidorus* species requires one additional restriction with *Fnu*4HI, while up to three subsequent cleavages are necessary for the identification of *Paratrichodorus* and *Trichodorus* species. The combination of the patterns obtained by up to four of seven individual enzymes allowed however, a clear differentiation of all species under analysis (Table 2).

Evaluation of the molecular diagnostic method (PCR-RFLP)

The repetitions performed with DNA extraction and the PCR-RFLP technique showed a high reproducibility of this technique. The restriction patterns of repeated samples processed in two occasions were indistinguishable.

The typability capacity of populations, previously morphobiometrically characterised, was 100%, regardless their geographic origin. However, two morphologically unidentified species (*Paratrichodorus* sp. C and D) were not clearly typified as any of the molecular type species. These two unidentified *Paratrichodorus* populations may represent distinct morphological species from those under analysis, and for which no molecular type species were included in the selected set of sequences.

The specificity of the test is mainly determined at the PCR step, due to lack of similarity between the 1671-F reverse primer and the selected sequences (*Longidorus elongates*, *Prismatolaimus intermedius*, *Pungentus silvestris*, *Pungentus* sp., *Thornia steatopyga*, *Tylencholaimellus striatus* and *Xiphinema rivesi*), that abrogate the amplification.

Sequences from other two genera, available in GenBank are incomplete not including the region that matches the reverse primer sequence: being therefore, impossible to foresee the specificity of the test applied to the complete 615bp sequences.

Discussion

In the present work we described a PCR-RFLP method that generates patterns for the clear identification of trichodorid species important for agriculture, including virus vector species. The restriction pattern produced by up to four of seven different enzymes segregated the twelve analysed species, nine of which are known to be TRV vectors.

The 18S rDNA region was selected as the target DNA to propose a molecular diagnostic technique for identification of trichodorids at species level. This region appears to be conserved within the populations of a trichodorid species, presenting however enough variability among species that generates a clear species identity profile. This complies with the known sequence variability higher among species, than among populations within species, due to concerted evolution of repeated DNA sequences (Dover, 1982). Other rDNA non-coding regions, such as the internal transcribed spacers, that are subjected to a lower selection pressure are frequently

used for diagnosis purposes. Non-coding regions have been reported as containing molecular polymorphism to allow distinguishing populations, for example, cyst nematode (Bekal *et al*., 1997, Szalanski *et al*., 1997, Subbotin *et al*., 2000, 2001 and Aimiri *et al*., 2002), *Pratylenchus* (Waeyenberg *et al*., 2000), and trichodorids (Boutsika *et al*., 2004). These authors differentiated four species, including *P. pachydermus* and *T. primitivus* from Scotland, UK, using species specific primers exploring the ITS variability.

The higher conservation of 18S region increases the difficulty of producing an easily differentiable band pattern required to facilitate the non-equivoques identification of the species. A single restriction enzyme does not allow the characterization of all investigated species; however, the discriminative power combined restriction enzymes, *Aat*II, *Ale*I, *Ava*I, *Bsr*I, *Fnu*4HI, *Taq*I and *Tsp*RI, resolved genomic groups among the trichodorid populations studied, although these species belong to three genera and differ by less than 6.6 % in their G+C content.

The use of well-characterized trichodorid populations by morphobiometry and by nucleotide sequence analysis allowed the validation of this PCR-RFLP for identification of trichodorid species. In the absence of previously defined and agreed molecular species types described by any of the present International Group, we define here, within the scope of the present work, the molecular type species analysed. The definition of molecular type species needs, in our opinion, future clarification to avoid potential confusions. This definition would also allow a credible comparison of nucleotide data, within populations of a morphological species.

When designing the PCR-RFLP, the priority was the proposal of a simple technique, easy to operate and inexpensive, which are basic requirements for a routine diagnosis technique. It was chosen a universal PCR reaction, to amplify a fragment with the shortest possible size (615nt) that, after a set of single digestions, produced bands easily differentiable in size. Single RE digestions can be performed successively, according to the flowchart of Figure 2, which allows the reduction of the number of digestions to a maximum of four, turning therefore the procedure less expensive. After the second restriction nearly half of the species may be identified and after

the third one nearly 84%. Furthermore, if time is a priority, the seven digestions can be set up simultaneously. Interpretation of results is quite straight forward.

The PCR-RFLP technique here described is the first molecular tool for trichodorids identification, discriminating populations of different species, but recognising identical populations of the same species. Such a method was long required for trichodorid and TRV management. Trichodorids do not move quickly or far, but once established in a soil they usually persist for many years and the virus they transmit tend to reappear at the same sites in successive plantings (Harrison & Robinson, 1986). Thereby, TRV management can be based on the assessment of vector trichodorid populations and their infectivity in soil samples collected before planting. The task of collect and processing soil samples and identifying trichodorid species requires trained staff, consequently, the existence of rapid, sensitive and species-specific nematological diagnostic methods is of the utmost importance for the effective risk assessment. This technique does not have to be performed by nematologists and is particularly adequate when only a few immature specimens are present in soil samples. In these cases, the incorrect identification can have major economical implications, as a few vector specimens are able to spread a compatible TRV isolate (Ploeg *et al*., 1992, Brown & Weischer, 1998). The method may be also important in the authentication and legal quarantine crop protection schemes as well as for other scientific studies.

The application of this PCR-RFLP method to different trichodorid populations originated from vaster geographic regions, or its bioinformatic simulation using new sequences when available in the GenBank, will allow further testing of the method. It will be then possible to verify if the proposed patterns are conserved, not only within the analysed populations, but also in others, from different countries or continents; or if, conversely, population variability will be detected, as it could be the case of *T. primitivus* from Azores.

The results here presented were very consistent. If the technique proves to be species-specific in laboratorial tests with vaster groups of soil-inhabitant nematodes, the method appears promising

to be performed directly with soil suspensions, avoiding the time consuming task of specimens' collection for DNA extraction.

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Table 1 Trichodorid populations and respective geographic origin, accession numbers of the 18S rDNA trichodorid sequences obtained from the EMBL/GenBank/DDBJ database used to design the PCR-RFLP method, and references of the populations assayed

Trichodorid populations	Geographic origin	Accession number of sequences	References[c] of tested populations
Nanidorus spp.			
N. minor[a]	Nm-Pt	DQ345526	Nm-Pt
N. minor		AM269897	
N. minor	Nm-Br		Nm-Br
N. nanus[a]		FJ040485/6	
Paratrichodorus spp.			
P. allius[a]		AM269895	
P. allius	Pall-US		Pall-US
P. anemones[a]	Pa-Gb-Eng	AF036600	Pa-Gb-Eng
P. divergens	Pd-Pt-a		Pd-Pt-a
P. divergens[a]	Pd-Pt-b	DQ345528	Pd-Pt-b
P. hispanus[a]	Ph-Pt	DQ345527	Ph-Pt
P. pachydermus[a]	Ppa-Gb-Sct	AF036601	Ppa-Gb-Sct
P. pachydermus	Ppa-Pt		Ppa-Pt
P. porosus[a]	Ppo-Pt	DQ345524	Ppo-Pt
P. porosus	Ppo-Br		Ppo-Br
P. teres[a]		AM269896	
Paratrichodorus sp. C[b]	PspC-Pt	DQ345523	PspC-Pt
Paratrichodorus sp. D[b]	PspD-Pt		PspD-Pt
Trichodorus spp.			
T. beirensis[a]	Tb-Pt	DQ345530	Tb-Pt
T. primitivus[a]	Tp-Pt	DQ345533	Tp-Pt
T. primitivus	Tp-Pt-Az		Tp-Pt-Az
T. primitivus	Tp- Gb-Sct	AF036609	Tp- Gb-Sct
T. lusitanicus[a]	Tl-Pt	DQ345532	Tl-Pt
Trichodorus sp. A[a]	TspA-Pt-a	DQ345534	TspA-Pt-a
	TspA-Pt-b		TspA-Pt-b
Trichodorus sp. B[a]	TspB-Pt	DQ345531	TspB-Pt

[a] Molecular type species for the purpose of the present work.

[b] Unidentified species.

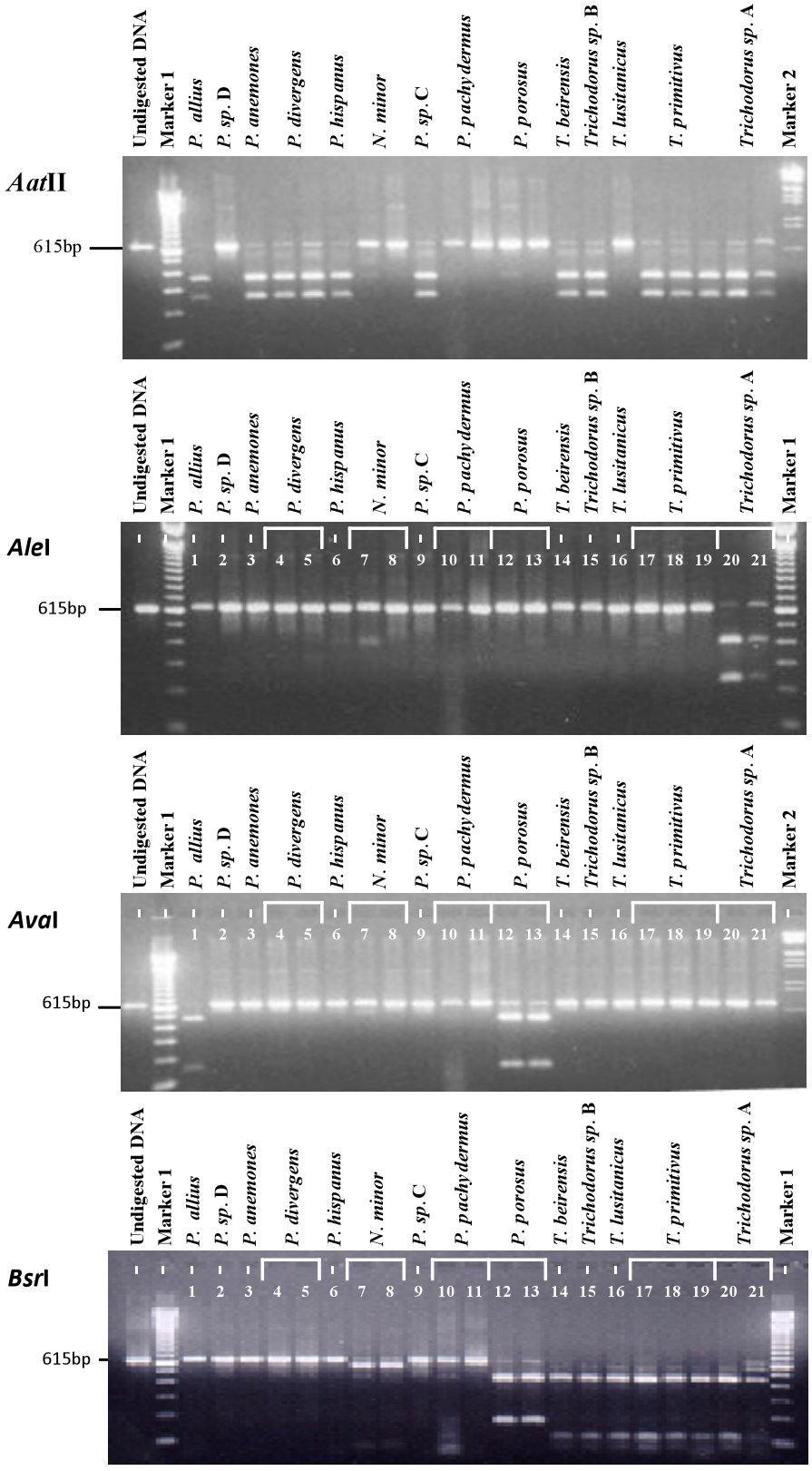
[c]Abbreviation criterion: first capital letter refers to genus, followed by one or two small letters referring to species-country of origin-additional letter to distinguish different sites.

Table 2 Restriction Fragment Length Polymorphism of the *ca*. 615 bp region of rDNA-18S for *Nanidorus*, *Paratrichodorus* and *Trichodorus* species. Expected sizes (base pairs) based on sequence information obtained from the EMBL/GenBank/DDBJ database

Trichodorid populations	*Aat*II	*Ale*I	*Ava*I	*Bsr*I	*Fnu*4HI	*Taq*I	*Tsp*RI
Nanidorus minor – Pt[a]	615	615	615	548, 67	275, 240, 40, 40, 17, 3	615	280, 220, 115
N. minor – Gr	615	615	615	548, 67	275, 240, 40, 40, 17, 3	615	280, 220, 115
N. nanus – Nl	615	615	615	548, 67	240, 215, 60, 57, 40, 3	615	280, 220, 115
Paratrichodorus allius – US[a]	358, 256	614	461, 153	614	302, 275, 37	614	282, 246, 86
P. anemones – Engl[a]	356, 256, 1	613	612, 1	612, 1	300, 275, 37, 1	529, 83, 1	332, 280, 1
P. divergens – Pt[a]	357, 257	614	614	614	301, 275, 38	360, 254	246, 220, 87, 61
P. hispanus – Pt[a]	358, 257	615	615	615	302, 275, 38	615	282, 246, 87
Paratrichodorus sp. C – Pt	358, 255	613	613	613	275, 241, 50, 36, 8, 3	246, 255, 112	282, 246, 85
P. pachydermus – Sct[a]	615	615	615	615	302, 275, 38	615	282, 246, 87
P. porosus – Pt[a]	614	614	461, 153	440, 174	302, 275, 37	614	282, 246, 86
P. teres – Gr[a]	614	614	614	614	302, 275, 37	556, 58	282, 246, 86
Trichodorus beirensis – Pt[a]	357, 258	615	615	442, 105, 68	301, 275, 39	615	334, 281
T. lusitanicus – Pt[a]	615	615	615	442, 105, 68	301, 275, 39	615	334, 281
T. primitivus – Pt[a]	356, 259	615	615	443, 105, 67	300, 192, 83, 40	615	280, 246, 89
T. primitivus – Sct	356, 259	615	615	443, 105, 67	300, 192, ([b]), 40	615	280, 246, 89
Trichodorus sp. A – Pt[a]	357, 258	392, 223	615	442, 105, 68	301, 275, 39	615	334, 281
Trichodorus sp. B – Pt[a]	357, 258	615	615	442, 105, 68	301, 192, 83, 39	615	334, 281

Engl, England; Gr, Greece; Pt, Portugal; Sct, Scotland; US, USA; [a] Molecular type species to the purpose of the present work; [b] a fragment of 83 nt is absent from this expected restriction fragments, when compared with the Portuguese sequence. This difference is due to the presence of 12 undetermined nucleotides in the Scottish *T. primitives* sequence (accession number, AF036609), abrogating the identification of a putative *Fnu*4HI restriction site.

1


Undigested DNA
Marker 1
P. allius
P. sp. D
P. anemones
P. divergens
P. hispanus
N. minor
P. sp. C
P. pachydermus
P. porosus
T. beirensis
Trichodorus sp. B
T. lusitanicus
T. primitivus
Trichodorus sp. A
Marker 2
AatII
615bp
Undigested DNA
Marker 1
P. allius
P. sp. D
P. anemones
P. divergens
P. hispanus
N. minor
P. sp. C
P. pachydermus
P. porosus
T. beirensis
Trichodorus sp. B
T. lusitanicus
T. primitivus
Trichodorus sp. A
Marker 1
AleI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
615bp
Undigested DNA
Marker 1
P. allius
P. sp. D
P. anemones
P. divergens
P. hispanus
N. minor
P. sp. C
P. pachydermus
P. porosus
T. beirensis
Trichodorus sp. B
T. lusitanicus
T. primitivus
Trichodorus sp. A
Marker 2
AvaI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
615bp
Undigested DNA
Marker 1
P. allius
P. sp. D
P. anemones
P. divergens
P. hispanus
N. minor
P. sp. C
P. pachydermus
P. porosus
T. beirensis
Trichodorus sp. B
T. lusitanicus
T. primitivus
Trichodorus sp. A
Marker 1
BsrI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
615bp

2

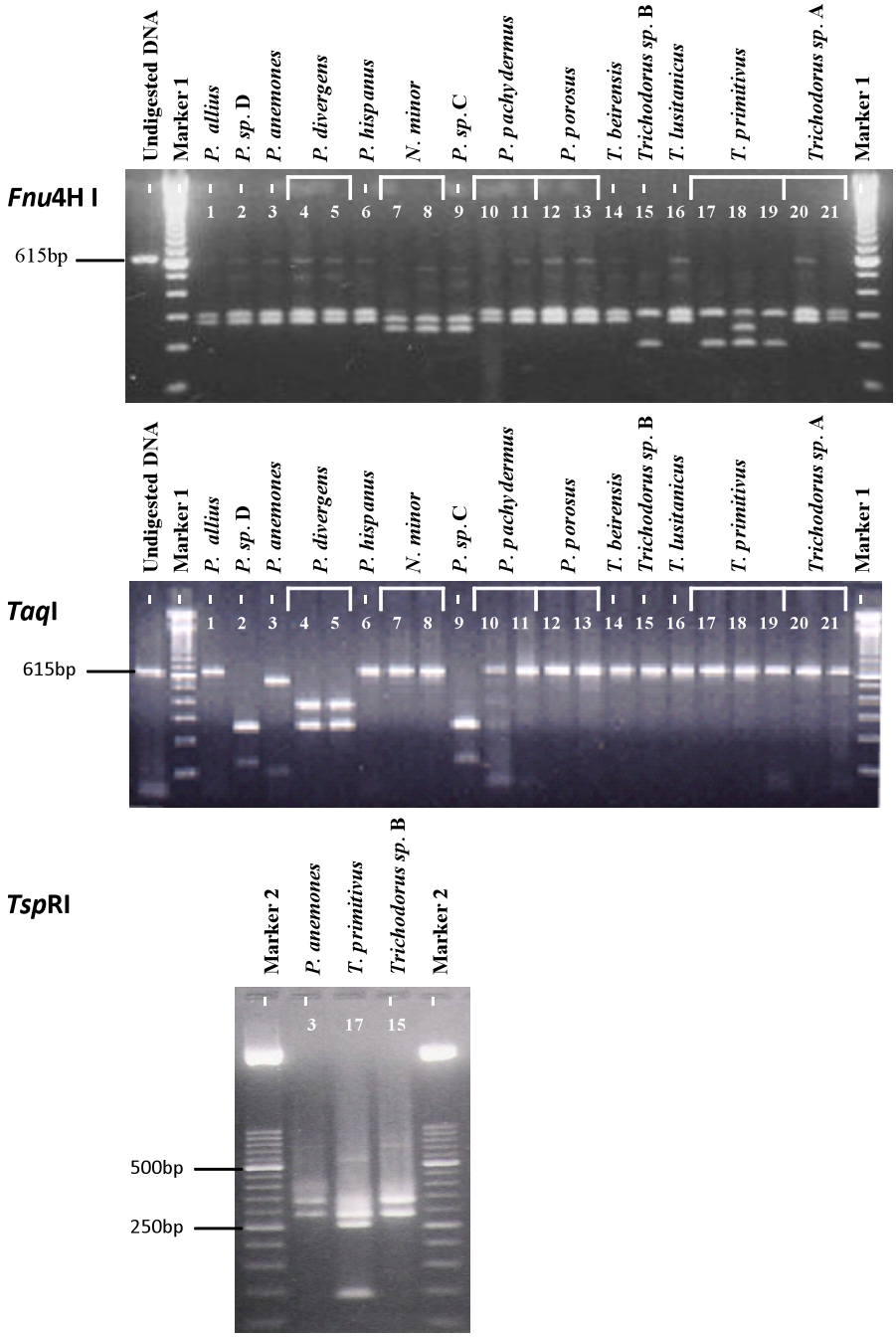


Figure 1 Restriction patterns of the PCR products enclosing the 3' end of 18S rDNA region of trichodorids obtained by simple digestion with *Aat*II, *Ale*I, *Ava*I, *Bsr*I, *Fnu*4H I, *Taq*I and *Tsp*RI. Code of the lanes: **(1)** *P. allius*, US; **(2)** *Paratrichodorus* sp. D, Pt; **(3)** *P. anemones*, Engl; **(4)** *P. divergens*, Pt-b; **(5)** *P. divergens*, Pt-a; **(6)** *P. hispanus*, Pt; **(7)** *N. minor*, Pt; **(8)** *N. minor*, Br; **(9)** *Paratrichodorus* sp. C, Pt; **(10)** *P. pachydermus*, Pt; **(11)** *P. pachydermus*, Sct; **(12)** *P. porosus*, Pt; **(13)** *P. porosus*, Bs; **(14)** *T. beirensis*, Pt; **(15)** *Trichodorus* sp. B, Pt; **(16)** *T. lusitanicus*, Pt; **(17)** *T. primitivus*, Pt; **(18)** *T. primitivus*, Pt-Az; **(19)** *T. primitivus*, Sct; **(20)** *Trichodorus* sp. A, Pt-a; **(21)** *Trichodorus* sp. A, Pt-b. *Tsp*RI patterns identical to those shown in lanes 3 and 17 were obtained

respectively with *Paratrichodorus* sp. D, Pt; *T. beirensis*, Pt; *T. lusitanicus*, Pt; *Trichodorus* sp. A, Pt-a; *Trichodorus* sp. A, Pt-b and *T. primitivus*, Pt-Az; *T. primitivus*, Sct; *P. allius*, US; *P. hispanus*, Pt; *Paratrichodorus* sp. C, Pt; *P. pachydermus*, Pt; *P. pachydermus*, Sct; *P. porosus*, Pt; *P. porosus*, Bs (results not shown). DNA molecular weight markers: Marker 1, 100 bp Ladder (Invitrogen, Life Technologies); Marker 2, XIII (50-750 bp), Roche, Diagnostic GmbH.

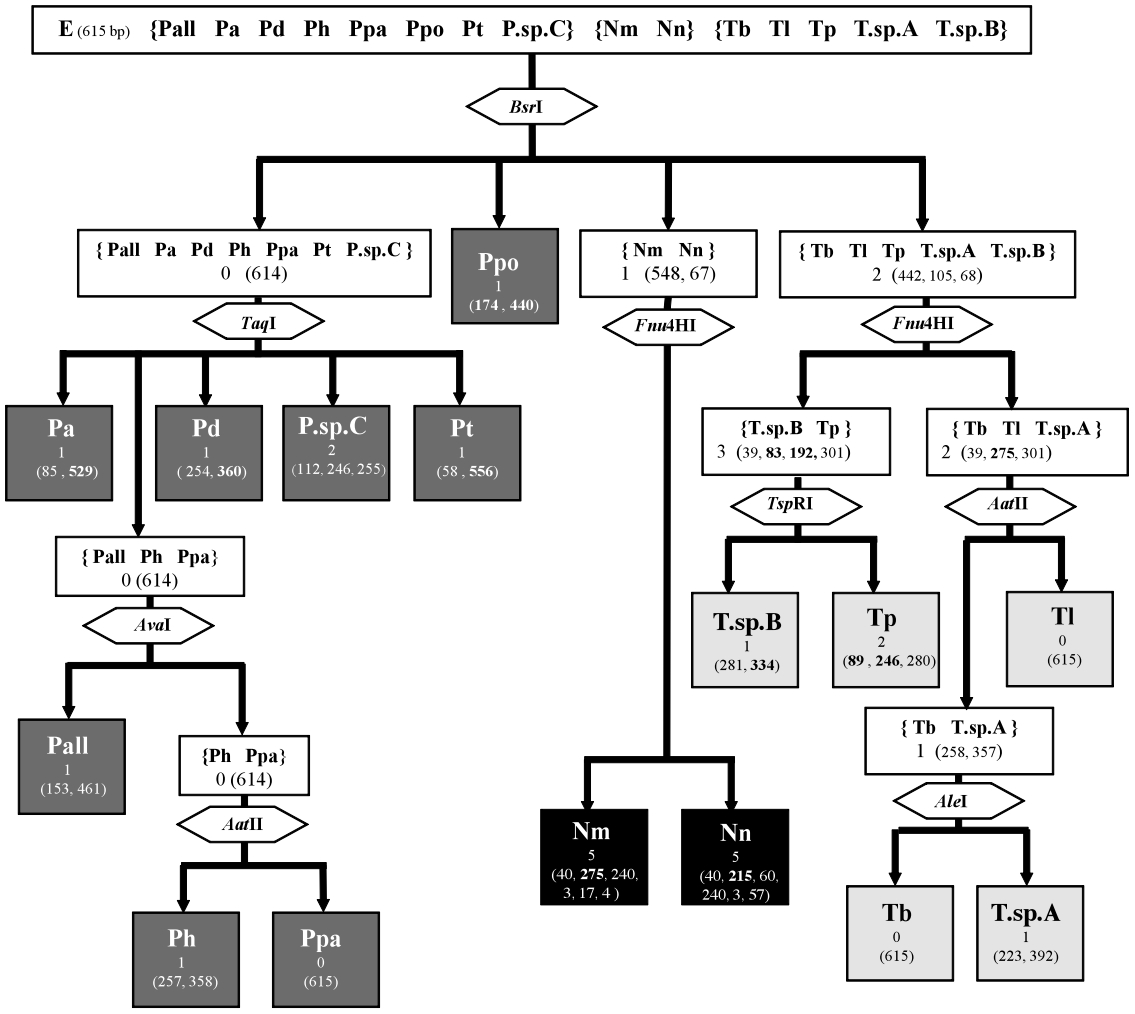


Figure 2 Flow chart summarizing the Restriction Enzyme digestions required for trichodorid identification to species level with PCR-RFLP technique. Rectangles, represent a group of indistinguishable species; hexagons, represent the digestions with restriction enzymes; Species identified are included in black (*Nanidorus*), dark grey (*Paratrichodorus*) and light grey (*Trichodorus*) squares: top line, species identified; middle line, number of targets for the RE indicated above; bottom line, sizes (bp) of the fragments produced. Nm, *N. minor;* Nn, *N. nanus;* Pall, *P. allius;* Pa, *P. anemones;* Pd, *P. divergens;* Ph, *P. hispanus;* Ppa, *P. pachydermus;* P.sp.C, *Paratrichodorus* sp. C; Ppo, *P. porosus*; Pt, *P. teres*; Tb, *T. beirensis;* Tl, *T. lusitanicus;* Tp, *T. primitivus;* T.sp.A, *Trichodorus* sp. A; T.sp.B, *Trichodorus* sp. B.

LEGENDS OF FIGURES

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Figure 1 Restriction patterns obtained by simple digestion of the PCR products enclosing the 3' end of 18S rDNA region of trichodorids.

Figure 2 Flow chart summarizing the Restriction Enzyme digestions required for trichodorid identification to species level.

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